EXHIBIT D

VACCINES AND IMMUNOTHERAPY

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Chapter 19

VACCINATION AGAINST RABIES

Reinhard Glück and *Marie Paule Kieny*

ETIOLOGIC AGENT AND PATHOGENESIS OF RABIES

Structure of Rabies Virus

Rabies virus is a member of the genus *Lyssavirus* within the family Rhabdoviridae, which contains more than 80 bullet-shaped viruses that infect vertebrates, invertebrates, and plants. Rhabdoviruses have a diameter of 70 to 80 nm and a length of approximately 180 nm. The genome of all rhabdoviruses consists of a single, nonsegmented, negative strand of RNA (approximately 4×10^6 Dal, 12 kb). The RNA is covered with approximately 1800 molecules of nucleoprotein (N), forming a nucleoprotein ribbon. Together with two additional proteins, the viral transcriptase (L) and the nonstructural protein (NS), the nucleoprotein is coiled into a cylindrical nucleocapsid (1). The nucleocapsid is surrounded by an envelope consisting of an inner layer of matrix protein (M) and a lipid bilayer through which peplomers [glycoprotein (G)] project (Figs. 1 and 2). Glycoprotein G is the only protein found on the outer surface of the virus (2). Rhabdoviruses have a sedimentation coefficient of approximately 600 S and a density of approximately 1.16 g/mL in sucrose gradients (3). The total chemical composition of rabies virus is approximately 1% RNA, 67% protein, 3% carbohydrate, and 26% lipid (4).

Antigenicity and Immunogenicity of Rabies Virus

Since the glycoprotein is the only rabies-specific antigen on the outer membrane of the intact virion and virus-infected cells, it has been generally accepted that this protein alone was responsible for the induction of protective immunity. Therefore, attention was focused primarily on this protein and its genomic sequence coding. Recently, attention has centered on the role of internal virion antigens in the establishment of protective immunity against viral infections by priming of cytotoxic T cells and induction of interferon (5–7). One reason for the continued interest in the role of internal rabies antigens was the observation that virus-neutralizing antibodies alone, induced by immunization with glycoprotein, did not confer complete protection against rabies virus challenge (8). Recent findings indicate that cytotoxic T cells induced by rabies infection or immunization with live or inactivated virus play a role in protective immunity (9). So far, two epitopes on the nucleoprotein N and an epitope on the nucleocapsid-associated protein NS that are recognized by cytotoxic T cells have been identified (10). Of these cytotoxic T cells, only those recognizing N epitope(s) have been implicated in protective immunity against disease (7).

Rabies glycoprotein obtained by treatment of purified virus with Triton X-100 and subsequent purification by isoelectric focusing is homogenous with respect to size and isoelectric point (2). Immunization with this material can provide good levels of protection against a lethal challenge of rabies virus. The molecular weight of such glycoprotein isolated under the above, denaturing conditions is approximately 400,000. Under denaturing conditions (SDS-polyacrylamide electrophoresis), only a single glycoprotein of M_r 67,000 is found. Therefore, it is conceivable that the peplomers of rabies virus are composed of hexamers of glycoprotein molecules. From the published sequence of cDNA clones of the glycoprotein gene of the ERA strain of rabies virus, it is known that there are four potential asparagine-linked glycosylation sites in the amino acid sequence, three

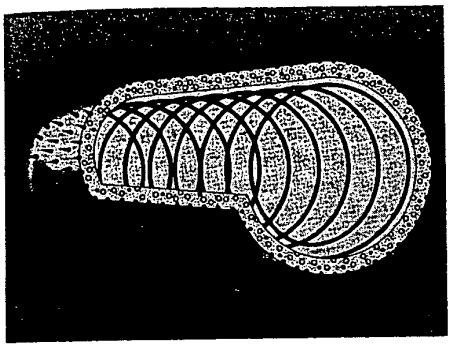

FIGURE 1. A schematic model of the rabies virus structure. The RNA is linked to about 1800 molecules of nucleoprotein to form a helical ribonucleoprotein that is tightly wound within the nucleocapsid. This is surrounded by a lipoprotein envelope (lipid + M protein) through which the surface spikes (glycoprotein) protrude. Two additional proteins, L and NS, are associated with the nucleolipid.

of which are located in the extramembranal part of the protein (11). The size of the oligosaccharide side chains varies, and it is possible that only two of the three potential sites are used (12). Purified rabies glycoprotein contains 11% oligosaccharides by weight. Although the exact function of the oligosaccharides has not been identified, several observations have been made that demonstrate their biological significance. First, it has been reported that inhibition of glycosylation results in a marked reduction of virus particle production (13). In addition, nonglycosylated glycoprotein produced by genetically engineered bacteria does not protect against a subsequent challenge with rabies virus (14).

The physical condition of rabies glycoprotein is important for its effectiveness as an antigen. Aggregates and soluble glycoprotein secreted by rabies-infected cells are poorly immunogenic (15, 16). Glycoproteins isolated from purified virus tend to aggregate. Aggregation may conceal protective epitopes and, therefore, diminish the amount of neutralizing antibodies induced. For this reason a commercially produced subunit vaccine elicited a significantly lower antibody titer in humans than intact virus vaccines. The soluble glycoprotein arises from normal, fully glycosylated G by in vivo cleavage of the transmembrane region. However, its protective ability is drastically reduced compared with that of full-length glycoprotein, because it has lost its capacity to aggregate. During the development of a highly purified vaccine from embryonated duck eggs, we observed that despite an unchanged glycoprotein content, the potency in mouse protection tests increased when the fractions with a density of 1.10 to 1.15 g/mL were added to the preparation that had fractionated at a density of 1.16 g/mL (authors' unpublished observations). These fractions contained a higher concentration of nucleoprotein than the high-density fractions. A role for the nucleoprotein antigen in protective immunity against rabies virus has been confirmed in more recent studies (7).

Pathogenesis of Rabies

Rabies infection is characterized by a sequence of events that almost invariably leads to death. The body reacts to the infection with several defense mechanisms that, even today, are not fully understood. Production of antibodies may even have a negative effect under certain circumstances. For example, it has been observed that when a rabies immune serum is given too late after exposure to rabies, it will induce an earlier manifestation of disease symptoms. This is called the *early death phenomenon* (17). A better understanding of the pathogenic mechanism would be advantageous so that a specific therapeutic treatment might be formulated.

Binding of rabies virus to susceptible cell surfaces is apparently not due to interaction with a single receptor molecule. Most likely, many cell surface components, particularly

FIGURE 2. Rabies virus in ultrathin section of infected BHK-21 cells × 150,000. Micrograph courtesy of M. Grassi, Swiss Serum and Vaccine Institute Berne.

lipids and lipoproteins, are involved in virus adsorption (18).

The neurotropic character of rabies virus has been described by many investigators (18). It seems, however, that other cell types are also infected, and their involvement plays an important role in disease progression. Upon entry of the rabies virus into susceptible cells (e.g., fibroblasts and neurons), lysosomal vesicles are involved in virus uncoating. After entry into the peripheral nervous system, rabies virus is transported to the central nervous system via the nerve fibers. An interruption of the axioplasmic flow by mechanical methods such as ligation or sectioning can prevent overt disease symptoms (19).

HISTORY OF VACCINATION AGAINST RABIES

In July of 1885, a 9-year-old boy was savagely bitten by a rabid dog. The wounds were so extensive that it was likely that the boy would succumb to rabies. Two days later in Paris, he was given the first of 13 injections of a rabies vaccine developed by Louis Pasteur that would save his life. It was the first time that a human condemned to die from rabies would be saved. During the following year, over 100 patients exposed to rabies were vaccinated by Pasteur, and the majority of them survived. In 1887, rabies vaccine was officially recognized by the medical community as an effective postexposure therapeutic measure (20).

The use of rabies vaccination is unique in that it has primarily been used as a therapeutic rather than a prophylactic agent, whereas most other vaccines are used prophylactically. It has been only during the past 10 years that vaccination against rabies has been applied as a routine prophylactic measure in certain high-risk populations.

In 1881, Pasteur identified a transmissible agent in the neurologic tissue of a rabies-infected dog (21). The disease could be transmitted by injecting healthy brains with neural material from rabies-infected tissue. The early Pasteur vaccines were prepared as follows: the virus was passaged in rabbits and reisolated from the spinal fluid after the paralytic phase of the disease (22); the virus was then "attenuated" by air drying, and after 9 to 14 days, fully inactivated virus was obtained. Pasteur's vaccination regimen called for a total of 13 injections administered daily following exposure; the first five with an "inactivated" preparation and the subsequent eight with an "attenuated" preparation.

Following these pioneering steps, numerous attempts were made in an effort to produce a vaccine by standardized techniques. E. Roux, in 1887, used glycerin to "attenuate" the virus. Subsequently, Fermi and Semple showed that phenol could be used to inactivate rabies virus (23, 24). Today, most rabies vaccines are inactivated with either formalin (25) or β-propiolactone (26), which consistently yield safe preparations.

Such early crude vaccines suffered from several drawbacks. Foremost was the fact that chemical inactivation with phenol or ether (27) did not, in all instances, result in total inactivation of the virus. Secondly, because these vaccines contained substantial amounts of foreign proteins, especially myelin basic protein, serious adverse reactions, such as encephalitis and anaphylaxis, occurred at a high frequency. In addition, neurologic complications, including paralysis, could occur at a frequency of 1 in 2000 patients (28). Finally, such vaccines were poorly immunogenic and may not have elicited a protective immune response even after repeated injections.

Rabies virus was first propagated in embryonated eggs by I. Kligler and H. Bernkopf in 1939 (29). In 1956, F. Peck described the production of a rabies vaccine wherein the virus was derived from embryonated duck eggs (12). The vaccine consisted of a homogenized duck embryo suspension inactivated by treatment with β-propiolactone; the suspension was lyophilized to increase stability. This vaccine, which contained only very low levels of basic myelin protein, had the advantage of inducing fewer neurologic reactions than brain-derived vaccines (30). However, it was also poorly immunogenic, requiring 17 daily injections to elicit a protective antibody response.

A major breakthrough in the preparation of an effective and complication-free vaccine was the development of new purification procedures and the propagation of virus on cultured cells. In the 1960s, it became possible to grow rabies virus in a variety of cell culture systems that yielded a virus suspension that was free from complex organ tissues. The first such rabies vaccine was produced in 1960 in primary hamster kidney cells (31). The virus was concentrated from the supernatant by ultracentrifugation and inactivated using formalin. Subsequently, a variety of other primary cells have been used to cultivate rabies virus. Vaccines produced in this manner have the distinct advantage of being essentially free of neurologic tissue. However, since pri-

mary cell culture techniques are used, fresh tissue must be obtained and grown for each vaccine lot. To avoid this problem, Wiktor (32) adapted the Pitman-Moore strain of rabies to human diploid cells, a finite-life cell line. Two such lines, termed Medical Research Control 5 (MRC-5) and Wistar 38 (WI-38), which can be passaged for roughly 60 generations without chromosomal alterations, have been certified as substrates for human vaccine production.

CURRENT ANTIRABIES PROPHYLACTIC AND THERAPEUTIC REGIMENS

Protection Against Rabies

Humoral antibody plays a central role in providing immunity against rabies. An antibody content of 0.5 IU/mL is considered to be protective. However, recent studies have shown that vaccine-induced cell-mediated immunity may also contribute to protection against disease.

In general, prophylactic immunization against rabies is recommended only for veterinarians, forestry workers, police, and laboratory workers with potential exposure to the virus. The number of vaccine doses and the immunization schedule depends upon the type of vaccine use (see below) and the geographic area. The majority of rabies vaccine is used following known or suspected exposure to the virus in combination with rabies immune globulin (33). It is critical to initiate treatment as soon as possible and to ensure that active and passive therapy be initiated simultaneously. Current recommendations call for the administration of 20 IU of human rabies immunoglobulin or 40 IU of heterologous (horse) immunoglobulin per kilogram of body weight, of which half should be injected into the wound site. It is important both that the potency of rabies immunoglobulin be determined by an appropriate test (mouse protection) and that the recommended amount of globulin not be exceeded because of the suppressive effect of excess antibody on vaccine-induced antibody formation.

Currently Available Vaccines

Human Diploid Cell Rabies Vaccine (HDCV)

The HDCV is considered the rabies vaccine of choice when measured by the criteria of safety, potency, and convenience of use. A major drawback to HDCV is its high cost, which precludes its use in developing areas. Rabies virus for vaccine production is grown on either the WI-38 or MRC-5 human diploid cell lines. The Pitman-Moore strain of fixed rabies virus is the most commonly used. Rabies virus is harvested from infected tissue culture, concentrated by filtration, and purified by sucrose density ultracentrifugation (34). The virus concentrate is adjusted to approximately $10^{7.5}$ tissue culture infectious dose 50 ($TCID_{50}$; equal to 5 IU of rabies antigen per milliliter) in a stabilizing solution. The virus is inactivated by the addition of β-propiolactone and the suspension lyophilized. Each final dose of vaccine must contain 2.5 IU as determined in a mouse protection test. The HDCVs are particularly attractive because they are free of contaminating nonhuman proteins that can induce serious allergic reactions. In addition, the rabies virus is purified from unwanted cellular debris.

Alternative Tissue Culture–Derived Rabies Vaccines

Several rabies vaccines are produced on nonhuman tissue culture substrates. Barth et al. (35) have described the production of vaccine from the Flury-LEP-C25 rabies virus strain on primary embryonated chicken cells (PCEC). Virus titers are routinely 10^7 $TCID_{50}$/mL or greater, making the vaccine economical. Viruses are concentrated and purified by density gradient ultracentrifugation and inactivated with β-propiolactone. Each dose of vaccine contains 2.5 IU of rabies antigen.

The world's largest producer, the Wuhan Institute in the People's Republic of China, grows the Beijing strain of rabies virus on primary Syrian hamster kidney cells (36). Two vaccine formulations are produced: one in which the viruses are purified by density gradient ultracentrifugation and another in which the crude harvested supernatant is mixed with an adjuvant. Both vaccines are inactivated by formalin.

The Pitman-Moore strain can also be grown on Vero cells, which are a continuous cell line of African green monkey kidney origin, in contrast to the above-described cell lines, which have a finite life in culture (37). Rabies virus is cultivated on Vero cells attached to microcarriers in 500- to 1000-L bioreactors. Virus is purified by ultrafiltration, continuous flow ultracentrifugation, and chromatography. Inactivation is by treatment with β-propiolactone.

Duck Embryo–Derived Vaccines

Purified Duck Embryo Vaccine (PDEV). Given the excellent yields of rabies virus obtained from embryonated duck eggs and the

continuing need for economical rabies vaccines in developing countries, techniques have been developed for purifying rabies virus from avian proteins (30). The PDEV is comparable to other rabies vaccines, including HDCV, in regard to safety and immunogenicity (38) and has the advantage of low production costs (39). Virus is extracted from the brain tissue of infected embryos in the absence of mechanical shear forces to reduce the release of soluble avian antigens. Purification is accomplished by density gradient ultracentrifugation and removal of nonviral lipids by extraction in a water-miscible organic solvent. The virus is inactivated with β-propiolactone. The final product contains no detectable myelin basic protein and only trace quantities of avian antigens (30). Of equal importance is the fact that the vast majority of rabies antigen consists of intact virus, not soluble glycoprotein, which has a markedly reduced immunogenicity (40).

Classic Duck Embryo Vaccine (DEV). The DEV has been employed since 1956 and is still used today in many areas of the world, where it has replaced the highly reactinogenic brain-derived vaccines. This vaccine is very economical and simple to produce. Embryos from infected eggs are harvested and homogenized in a stabilizing solution. The homogenate is clarified by filtration and centrifugation followed by treatment with β-propiolactone. There are two major drawbacks to the use of this vaccine. First is the fact that 14 primary injections and 3 booster doses are required (see below). Second is the high rate of adverse reactions, some with neurologic sequelae, due to the large quantity of avian proteins, especially myelin basic proteins, contained in the vaccine.

Neural Tissue–Derived Vaccines

Suckling Mouse Brain Vaccine (SMBV). The SMBV was developed in Chile in 1954 (41) and is still used, particularly in South America. Three fixed virus strains (Chile 51, Chile 91, or the CVS strain) are used to inoculate mice. The brains are harvested and inactivated with phenol and preserved in thimerosal. Each human dose contains roughly 20 mg of murine brain tissue and 1.3 IU of rabies antigen. This vaccine, although crude, is protective when multiple doses (10) are administered and produces fewer neurologic complications than vaccine derived from adult animal brains (see below).

Semple-Type Vaccine. The Semple-Type vaccine is prepared from rabies-infected brain tissue derived from rabbits, sheep, or goats. The PV or CVS strain of rabies virus is used. A brain suspension is produced by homogenization, and inactivation is accomplished by the addition of phenol (28). One human dose consists of 2 mL of a 5 to 10% brain suspension. The vaccine is highly reactinogenic but is still used in certain developing countries because of ease of manufacturing and low cost.

Immunization Schedule

With new-generation vaccines of increased antigenicity and immunogenicity, attention has focused on reducing the number of vaccine doses needed to obtain a protective immune response within a short period of time (Table 1). Vaccines of weak potency, such as the DEV and brain-derived vaccines, are administered daily for 10 to 14 consecutive days. Since 1978, more potent vaccines (≥2.5 IU/dose), such as HDCV, PDEV, and nonhuman cell–derived vaccines, have been given by an abbreviated schedule with immunizations on days 0, 3, 7, 14, 30, and 90 (34). Recently, it has been shown that an optimal response is obtained by the administration of two doses of vaccine at two anatomic sites, usually both deltoid regions, on day 0 followed by injections on days 7 and 21 (42). This immunization regimen has been shown to be effective for several potent rabies vaccines and is now recommended by the WHO. In instances in which vaccine availability is restricted, smaller quantities can be given by the intradermal, instead of the usual intramuscular, route (43).

PROSPECTS FOR FUTURE RABIES VACCINES

Progress during the past decade in the fields of molecular biology and immunology has opened new vistas in the development of safer, more effective and more economical vaccines against rabies. The ultimate goal is to devise a one-dose vaccine that can be manufactured at a reasonable cost. Efforts have centered around inducing both a humoral and a cell mediated immune (CMI) response to the viral surface glycoprotein (7).

Live-Attenuated Rabies Virus Strains

Based upon the remarkable success using live-attenuated vaccines against other viral diseases, efforts have been made to isolate

TABLE 1. Composition of the "New Generation" Rabies Vaccines

Vaccine	*Producer*	*Virus Substrate*	*Antigenic Value (IU/Dose)*	*Rabies Strain*
Human diploid cell rabies vaccine (HDCV)	Behringwerke, Connaught Lab., Institut Mérieux, Swiss Serum and Vaccine Institute Berne	WI-38 cells MRC-5 cells	> 2.5	PM, ERA
Purified chicken embryo cell rabies vaccine (PCEC)	Behringwerke	Primary chicken fibroblasts	> 2.5	Flury-LEP C25
Purified VERO rabies vaccine (PVRV)	Institut Mérieux	VERO cells from ATCC at PD124	> 2.5	PM
Purified duck embryo rabies vaccine (PDEV)	Swiss Serum and Vaccine Institute Berne	Embryonated duck eggs	> 2.5	PM

avirulent or attenuated strains of rabies virus. Due to the lethal nature of rabies infection, live-attenuated rabies vaccine research has, therefore, been restricted to the veterinary field to avoid vaccine-associated disease. The attenuated Flury-HEP strain had been used for quite some time to vaccinate dogs but has recently been withdrawn due to the danger of reversion to virulence. For oral immunization of foxes in central Europe, attenuated virus strains like SAD/BHK 21 (44) or SAD B 19 are still used (45). These strains elicit protective levels of antibody in animals, but they are not pathogenic for humans. In addition, they can be distinguished from wild-type strains by markers expressed in vitro (46).

Several laboratories have tried to isolate attenuated variants of rabies virus by virtue of their resistance to neutralization with monoclonal antibodies (47–49). Several such variants harbored a mutation at the same site in the glycoprotein (Arg-333) (12). Although such mutants provided good protection in animal experiments, their application as a human vaccine is doubtful, since such viruses in rodents show a tendency to revert after passage (12).

Synthetic Peptides

Although synthetic peptides have shown very promising results in other systems (50), results so far with peptides encoding various regions of rabies virus glycoprotein have been disappointing. A large number of synthetic peptides have been tested for their immunologic responses. Out of 26 peptides spanning the majority of the glycoprotein sequence and selected on the basis of hydrophilicity, only two were able to stimulate T-cell proliferation. Additionally, induction of virus-neutralizing antibody was poor, and no protection against challenge was observed (12). These negative results are reminiscent of the poor responses observed with the soluble glycoprotein synthesized by rabies-infected cells (16).

Antiidiotypic Antibodies

Studies with purified rabies glycoprotein or synthetic peptides indicate that loss of conformational epitopes are responsible for the poor protective capacity of these vaccine candidates. Therefore, a soluble macromolecule that mimics the secondary and tertiary structures of the native glycoprotein would be an ideal vaccine antigen. Antiidiotypic antibodies could, in theory, fulfill these requirements. Antiidiotypic antibodies against several monoclonal anti-G antibodies were induced with the aim of generating a population of internal image antibodies that would mimic the epitope on native glycoprotein to which the monoclonal antibody binds (51). The antisera raised in rabbits against five such mouse monoclonal antibodies were tested in mice. These antibodies, however, did not

confer protection against a lethal dose of challenge virus.

Until a convenient in vitro source of human immunoglobulins has been developed, antiidiotypic antibodies against rabies virus proteins will have relatively poor prospects for use as a vaccine in humans.

Production of Rabies Glycoprotein by Genetic Engineering

Several groups have attempted to circumvent the need to produce intact virus as vaccine antigen by using recombinant DNA technology, the aim being to obtain large quantities of rabies glycoprotein from microorganisms or mammalian cell cultures. The coding sequence of rabies glycoprotein has been obtained from the ERA strain (52) as well as from the CVS strain (53). These sequences were expressed in large quantities in *Escherichia coli* (53, 54). Although these polypeptides reacted with antisera against native glycoprotein, they were unable to protect against a lethal dose of challenge virus. Presumably, the cause of the failure lies in the abnormal folding of the recombinant G protein as well as in the lack of glycosylation. In addition, it was observed that such proteins failed to induce a CMI response.

Unlike procaryotes, the yeast, *Saccharomyces cerevisiae*, is a eucaryote and able to glycosylate polypeptides via asparagine linkages (55). Several groups, therefore, have tried to express rabies G protein in this yeast system (12). Although it was demonstrated that the expressed G protein was glycosylated, the protective capacity of this recombinant glycoprotein is unknown at this time. Additional possibilities for the expressive of G protein exist with the use of eucaryotic cell lines. In this system, a correctly folded and glycosylated G protein was transported to the cell surface (12). Whether this molecule can provide protection against experimental rabies is unknown at this time.

It was demonstrated earlier that G protein synthesized in vitro could be inserted into vesicles of the glycoside Quil-A, forming an immunostimulating complex (ISCOM) (56). The protective immunity provided by such ISCOMs was comparable to that induced by standard rabies vaccines. It is possible that recombinant DNA technology combined with Quil-A manufacturing technology could provide an efficacious and safe vaccine against rabies infection. The safety and immunogenicity of such vaccines have not yet been evaluated in humans.

Expression of Rabies Virus Glycoprotein in a Vaccinia Virus Vector

Live-attenuated vaccinia virus (VV) vaccine has been used successfully to eradicate smallpox. Its stability, ease of administration, and low cost were greatly beneficial in vaccination campaigns in less-industrialized countries. Both Moss and coworkers (57) and Paoletti and coworkers (58) were the first to use VV (a large DNA virus with a fair part of the DNA being nonessential for virus replication and, therefore, available for insertion of foreign DNA sequences) for the expression of exogenous DNA coding sequences. Since then, this vector has been used extensively for expression of cloned antigens from hepatitis B, influenza, vesicular stomatitus virus, and *Plasmodium knowleri* (simian malaria), for example. Similarly, the coding sequence of the ERA rabies strain glycoprotein was introduced into the genome of the Copenhagen strain of VV (59) (Fig. 3). Infection of cultured cells with recombinant viruses resulted in the production of an apparently correctly processed rabies G protein, which reacted with virus-neutralizing monoclonal antibodies. The reaction profile of such glycoprotein with a panel of monoclonal antibodies largely paralleled the profile observed with native ERA G protein on virus (60). The immunogenic properties of this VV were tested in animal experiments (60). Intradermal inoculation of rabbits and mice resulted in a rapid induction of rabies virus–neutralizing antibodies. All mice were protected against a lethal challenge dose of CVS virus. The rabbit sera showed neutralizing activity against the ERA strain of rabies virus and against Duvenhage bat virus and three stains of CVS virus as well.

Since a large part of the world's population has been previously vaccinated with live VV (smallpox vaccine), the possible interference of an earlier smallpox vaccination with an immunization employing an engineered VV has been studied in a rabbit model (60). When the same engineered VV was used for initial immunization and for reinfection 5 months later, it was shown that the high neutralizing antibody titers at the time of reinfection increased rapidly and that animals were protected against a lethal challenge with rabies virus.

Engineered VV containing the rabies gly-

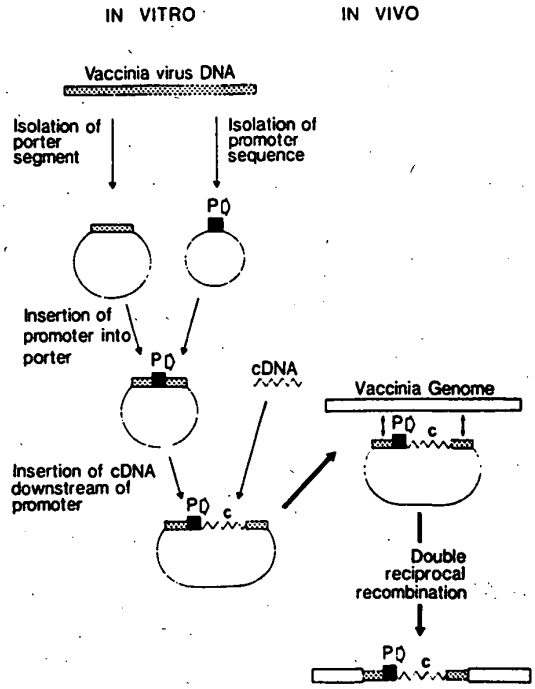

FIGURE 3. Summary scheme for the cloning and expression of an exogenous protein-coding sequence in a vaccinia virus vector. Constructions on the left are performed in vitro, whereas transfer to the vaccinia genome by recombinant exchange (right) is performed in vivo.

coprotein has the advantage that it induces rabies-neutralizing antibodies irrespective of the route of inoculation: intramuscular, intradermal, and oral routes are all effective. Experiments in raccoons in the United States and in foxes in Europe (60) showed that vaccination conferred a high level of protection against an experimental challenge. Hamsters were inoculated with recombinant VV before and after challenge with a high dose of rabies virus. The level of protection obtained in this case was comparable to or better than the level induced with a conventional rabies vaccine. Such experiments demonstrate that the recombinant VV is at least as effective in inducing protective immunity as conventional rabies vaccines in animal trials. Its application as a human vaccine has been delayed to date based upon the incidence of rare cases of VV vaccine–associated complications in the smallpox vaccination campaign. Nevertheless, thymidine kinase–deficient VV mutants have been shown to be markedly attenuated compared with the conventional VV vaccine strain and hold much promise as a safe carrier for rabies glycoprotein (60).

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